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Enteric neuroplasticity in seawater-adapted European eel (*Anguilla anguilla*)

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Abstract

European eels live most of their lives in freshwater until spawning migration to the Sargasso Sea. During seawater adaptation, eels modify their physiology, and their digestive system adapts to the new environment, drinking salt water to compensate for the continuous water loss. In that period, eels stop feeding until spawning. Thus, the eel represents a unique model to understand the adaptive changes of the enteric nervous system (ENS) to modified salinity and starvation. To this purpose, we assessed and compared the enteric neuronal density in the cranial portion of the intestine of freshwater eels (control), lagoon eels captured in brackish water before their migration to the Sargasso Sea (T0), and starved seawater eels hormonally induced to sexual maturity (T18; 18 weeks of starvation and treatment with standardized carp pituitary extract). Furthermore, we analyzed the modification of intestinal neuronal density of hormonally untreated eels during prolonged starvation (10 weeks) in seawater and freshwater. The density of myenteric (MP) and submucosal plexus (SMP) HuC/D-immunoreactive (Hu-IR) neurons was assessed in wholemount preparations and cryosections. The number of MP and SMP HuC/D-IR neurons progressively increased from the freshwater to the salty water habitat (control > T0 > T18; $P < 0.05$). Compared with freshwater eels, the number of MP and SMP HuC/D-IR neurons significantly increased ($P < 0.05$) in the intestine of starved untreated salt water eels. In conclusion, high salinity evokes enteric neuroplasticity as indicated by the increasing number of HuC/D-IR MP and SMP neurons, a mechanism likely contributing to maintaining the body homeostasis of this fish in extreme conditions.

Key words: enteric neuroplasticity; immunohistochemistry; neurogenesis; neuronal migration.

Introduction

The European eel (*Anguilla anguilla*) is a catadromic teleost that lives most of its life in freshwater of lakes and rivers until its spawning migration to the Sargasso Sea. During seawater adaptation the eel modifies not only its skin pigmentation, which changes from yellow to silver, but also its physiology, to adapt to the new saline environment. New metabolic functions must support the increased intake and intestinal absorption of seawater. Some structures, such as the digestive system and the gills, need to regulate body homeostasis to the different salinity of the water ingested (Laverty & Skadhauge, 2012). Furthermore, before leaving the lagoons, the eel needs to eat more food to increase its fat stores to support the energy requirements during its long migration (5000–6000 km) to the Sargasso Sea. It seems, in fact, that the eel stops feeding until spawning (i.e. even up to a few months), which causes some morphological modifications of its body, such as progressive slimming and reduction of the masticator muscles. Once it reaches the reproductive area of the Sargasso Sea, the eel dies after spawning as a result of the extreme effort.

This still mysterious biological life cycle has hampered its reproduction in captivity; in fact, its biological features have attracted the interest of researchers over the centuries (Redi, 1684). Currently, some laboratories are able to induce eels to sexual maturity, egg production and fertilization after 18 weeks of starvation and hormone treatment (see below) (Mordenti et al. 2012a,b) – the latter being crucial to bring eels to complete sexual maturity. Thus, the eel represents a unique model to understand the adaptive changes

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of digestive innervation, i.e. the enteric nervous system (ENS), to modified salinity, starving, and hormonal treatment.

In this study, we planned to investigate the adaptive changes of eel ENS during the long period of starvation, adaptation to saltwater, and hormonal treatment. Specifically we aimed to evaluate the density of ENS neurons, in cryosections and wholemount preparations of freshwater and salt-adapted starved eels, with or without hormonal treatment.

Material and methods

All the procedures described below were carried out in accordance with the European Communities Council Directive (86/609/EEC 24 November 1986) and the Italian legislation regarding experimental animals, after approval by the Scientific Ethics Committee for Experiments on Animals of the University of Bologna (Protocol 2-IX/9, All. 31).

The experiments were organized in two steps. First, we investigated the modification of the eel ENS during prolonged starvation in seawater following hormonal treatment with standardized carp pituitary extract (CPE). Intestinal specimens from control freshwater and starved seawater-adapted eels were obtained and assessed. Animals were classified as control, T0, and T18.

Control eels (n = 10; three females and seven males) aged about 4 years (male) and 5 years (female) (age estimated by the observation of the inner ear bones) were selected from a local fish farm and subsequently raised in freshwater and normally fed.

T0 eels (n = 11; 11 females) were captured in three different salty water lagoons in northern Italy before leaving the coasts for reproductive migration. These eels were not a homogeneous population in terms of length and weight. It is unknown whether these subjects, already prepared for their long migration, were already in a starvation state. T0 eels were sacrificed one or 2 days after being captured.

T18 female eels (n = 6) were captured in salty water lagoons in northern Italy (as T0 eels) and were adapted to seawater, in captivity, with salinity of 28–33‰ and 15.5 ± 0.5 °C temperature in total darkness. T18 eels were registered and individually marked with a cutaneous tag (Floy®-T-Bar Anchor, Floy Tag, Union Bay Place NE, Seattle, WA, USA); they were then treated, after sedation with MS-222 (tricaine methanesulfonate; 80 mg L^{-1}), with increasing doses of carp pituitary extract (CPE; Allwells Marketing Ltd, Bangladesh) by weekly intramuscular injections (18 injections of 10, 20, 30 and 40 mg kg^{-1} BW) to induce sexual maturity (Mordenti et al. 2012a, b). The final oocyte maturation and ovulation was induced by injection of 17,20 h-dihydroxy-4-pregnen-3-one (DHP) at 2 mg g^{-1} BW (Mordenti et al. 2012a,b).

The preliminary results obtained from the first step of this research demonstrated an unexpected increase in neuronal density of seawater-adapted, hormone-treated eels; therefore, in the second step of our experiment we analyzed the effects of salinity (and starvation) on the ENS of eels that had not undergone hormone treatment. We used eight male starved eels maintained for 10 weeks in salty (n = 4) and fresh (n = 4) water. As with the first group, these subjects were also registered and individually marked with cutaneous tag. The salinity of the seawater was the same as described above. Both groups of males were kept in two independent 70-L aquariums, each with mechanical and biological filtering, at a controlled temperature of 15.5 ± 0.5 °C and for 24 h per day without light (−0.04 × 10^3 lux at the bottom of the aquarium without water).

All the data related to the subjects employed are listed in Table 1.

Tissue preparation

Deeply anaesthetized eels (Mordenti et al. 2012a,b) were euthanized by decapitation performed behind the pectoral fins. The retropharyngeal digestive apparatus was removed from the esophagus to the anus after a longitudinal incision of the abdomen. The intestine was quickly measured (from the pylorus sphincter to the anus) and immersed in phosphate-buffered saline (PBS; 0.15 M NaCl in 0.01 M sodium phosphate buffer, pH 7.2) containing the L-type calcium channel blocker nicardipine (1026 M; Sigma-Aldrich Chemie, Steinheim, Germany) for 30 min. The oral pole of the esophagus and the caudal pole of the intestine were pinned with entomological pins on a balsa wood (2.5 mm thickness); the intestine was dissected from the adjacent stomach and then cut open along the mesenteric border under a stereomicroscope (*Zeiss* KL 1500 LCD). Tissues were then pinned tautly onto balsa wood, mucosal surface facing up, with entomological brass pins (Australian Entomological Supplies P/LTD, 14 × 0.55 mm). The specimens were subsequently fixed in Zamboni's fixative (2% paraformaldehyde containing 0.2% picric acid in 0.1 M sodium phosphate buffer, pH 7.0) at 4 °C overnight. When removed from the balsa wood they were washed in dimethylsulfoxide (DMSO; 3 × 15 min), and then in PBS (3 × 15 min). All tissues were stored at 4 °C in PBS containing sodium azide (0.1%) until they were processed to obtain wholemount preparations or cryosections.

Cryosections

To obtain frozen tissues, small longitudinal portions (1 × 0.3 cm) of cranial intestine were stored at 4 °C in PBS containing 30% sucrose and sodium azide (0.1%). The following day, tissues were placed in a mixture of PBS–30% sucrose-azide and Optimal Cutting Temperature (OCT) compound (Sakura Finetek , Zoeterwoude, NL, Europe) at a ratio of 1 : 1 for an additional 24 h before being embedded in 100% OCT in Cryomold® (Sakura Finetek, Zoeterwoude, NL, Europe). Sections were prepared by freezing tissues in isopentane cooled in liquid nitrogen. Serial longitudinal sections (14–16 μm thickness) of tissues were cut on a cryostat (Leica) and mounted on polysine-coated slides. Sections were stored at −80°C until the histological and/or immunohistochemical experiments were started.

Table 1 Gross anatomy data of the subjects employed.

	Body weight, g	Body length, cm	Intestinal length, cm
Control ♀ (n = 3)	521 ± 136	62 ± 4	15 ± 2
Control ♂ (n = 7)	140 ± 10	41 ± 3	9 ± 1
T0 ♀ (n = 11)	480 ± 242	65 ± 8	14 ± 3
T18 ♀ (n = 6)	788 ± 430	71 ± 8	19 ± 5
Seawater starved ♂ (n = 4)	80 ± 20	39 ± 3	8 ± 0.5
Freshwater starved ♂ (n = 4)	77 ± 13	39 ± 3	8 ± 0.4

Wholemount

The intestinal specimens (cranial half of the intestine) were pinned flat using thin pins, with mucosa side up, in a Sylgard-covered Petri dish containing PBS. Theoretically, the term 'submucosa' should not be used for eels, as their gut lacks a *muscularis mucosae*; nevertheless, for the sake of clarity, we applied the term 'submucosal neurons' and/or submucosal plexus (SMP) throughout the text to indicate the neurons lying above the myenteric plexus. To obtain a wholemount preparation of myenteric plexus (MP) it is necessary to remove, with fine forceps (Electron Microscopy Sciences, Dumoxel, Biology grade # 5), the circular muscle layer (CML).

Measurement of muscle layer thickness

The thickness of the circular and longitudinal muscle layers (CML and LML, respectively) of eel intestine ($n = 3$ for each group) was measured in longitudinal cryosections, following manual tracing (AXIOVISION software, Carl Zeiss, Oberkochen, Germany). For T0 eels, we used one animal for each of the three different environments where they were captured (see above).

For each eel, three measurements were performed in three different positions of the same section and the average considered for the evaluation.

Data are presented as mean values ± standard deviation. The statistical analysis was performed by means of one-way ANOVA (SPSS 17.0, SPSS Inc., Chicago, IL) combined with Tukey's post-hoc (HSD) test. The level of significance was set at $P < 0.05$.

Immunohistochemistry

Single labelling studies were performed using the indirect immunofluorescence method. To reduce background staining and enhance the penetration of the antibodies into tissues, 10% of appropriate normal goat serum (NGS) and 0.5% Triton-X100, respectively, were used in the pre-incubation stage and also in all antibody solutions. Tissues were incubated in 10% NGS in PBS, for 1 h, at room temperature (RT).

Cryosections were then incubated overnight in a humid chamber at RT in the antibody solution; wholemount preparations were incubated overnight in a multi-well chamber agitated by a rotator at RT. The primary antibody (mouse anti-Hu, 1:400, code A21271, #833294, Molecular Probes, Eugene, OR) was diluted in 1.8% NaCl in 0.01 M sodium phosphate buffer containing 0.1% sodium azide. After washing in PBS (3 × 10 min), the tissues were incubated for 1 h (cryosections) and 3 h (wholemounts) at RT in a humid chamber with the secondary antibodies (goat anti-mouse IgG Alexa Fluor® 594, 1 : 200, Molecular Probes) diluted in PBS. The cryosections and wholemount preparations were then washed in PBS (3 × 10 min) and mounted in buffered glycerol at pH 8.6.

The correlation between enteric glial cells and enteric neurons was analyzed in all five groups by double-labelling studies performed on cryosections. After the pre-incubation stage, sections were incubated overnight in a humid chamber at RT in a mixture of two primary antisera – mouse anti-Hu (see above) and rabbit anti-S100β (1 : 300; code Z0311, # 00084964, Dako Cytomation, Golstrup, Denmark) – diluted in 1.8% NaCl in 0.01 M sodium phosphate buffer containing 0.1% sodium azide. After washing in PBS (3 × 10 min), tissues were incubated for 1 h at RT in a humid chamber in a mixture of two secondary antibodies, i.e. goat anti-mouse IgG Alexa Fluor® 594 (see above) and goat anti-rabbit FITC (1 : 200; Calbiochem-Novabiochem, San Diego, CA). The cryosections were then washed in PBS (3 × 10 min) and mounted in buffered glycerol at pH 8.6.

Diaminobenzidine immunohistochemistry

After incubation in the primary mouse anti-Hu antibody in a humid chamber at 4 °C (as described above), cryosections were mounted on polysine-coated slides and incubated 2 h at RT with biotinylated goat anti-mouse IgG, 1 : 200 (Vector Laboratories). After being washed (3 × 10 min) in PBS, the specimens were treated for 45 min at RT with the avidin-biotinperoxidase-complex (ABC kit; Vector). After three washes (10 min each) in PBS, immunoreactive sites were visualized by treating tissues for 3 min with 3,3′-diaminobenzidine (DAB kit; Vector). Immunostained specimens were dried overnight at RT, quickly dehydrated in ethanol, cleared with xylene, and coverslipped with Entellan (Merk, Darmstadt, Germany).

Fluorescence microscopy

Preparations were examined on a Zeiss Axioplan microscope (Axioplan epifluorescence microscope, Carl Zeiss, Oberkochen, Germany) equipped with the appropriate filter cube to distinguish the fluorochrome employed: Alexa 594 (530–585 nm excitation filter and 575–615 nm emission filter). The images were recorded with a Zeiss Axiocam MRm (Carl Zeiss, Oberkochen, Germany) and AXIOVISION software. Slight adjustments to contrast and brightness were made using Adobe PHOTOSHOP CS, whereas the figure panels were prepared using Corel DRAW.

To evaluate the density of MP neurons in wholemount preparations, the number of Hu-IR neurons was counted in 10 randomly chosen microscopic fields (magnification 40×) for each eel; each field measured 164 × 219 μm, i.e. 35 916 μm^2 (10 fields = 359 160 μm^2). Neuronal density of MP and SMP was evaluated by counting Hu-IR neurons in 1-cm-long longitudinal cryosections. Data are presented as mean values ± standard error of the mean (SEM). The level of significance was set at $P < 0.05$.

Since the MP neurons observed in wholemount preparations appeared to belong mainly to three categories of neurons, small- (the majority of them), medium-, and very large-sized ('giant neurons') (Fig. 2), we evaluated the cross-sectional area of 300 neurons (at least 100 neurons of each size category) in three eels from each of the five groups. The cross-sectional area was measured after manual tracing of the stained perikarya (AXIOVISION software). Only cells in focus were chosen for measurements, which yielded a total of 1500 cells. Data are presented as mean values ± standard deviation. The level of significance was set at $P < 0.05$.

Results

Measurement of muscular layer thickness

In freshwater (control) eels, CML thickness was 316 ± 107 μm whereas for LML it was 95 ± 18 μm, with a CML/LML ratio of 3.3. CML and LML thicknesses of control eels was compared with that of the muscle layers measured in the other groups. In T0 eels, i.e. subjects ready for migration to the Sargasso Sea, CML thickness was increased (331 ± 212; $P > 0.9$), whereas LML thickness was decreased (57 ± 10 μm; $P > 0.1$) (CML/LML = 6.3; $P > 0.4$). After 18 weeks of starva-

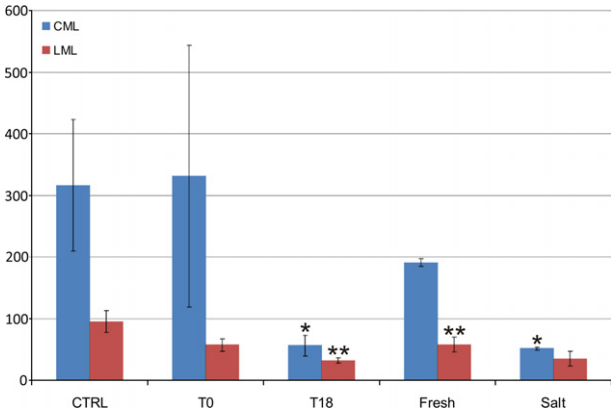

Fig. 1 Graphical representation of the thickness of the circular muscle layer (CML) and longitudinal muscle layer (LML) of the cranial portions of the intestine of the following groups of eels: CTRL (control) eels, female freshwater subjects; T0 eels, female subjects captured in salty water lagoons in northern Italy before leaving the coasts for reproductive migration; T18 eels, female eels captured as T0 subjects and adapted in captivity to seawater salinity for 18 weeks. Fresh and salt eels were male hormone-untreated subjects maintained in starvation for 10 weeks in freshwater and seawater, respectively. Data are expressed as mean ± standard deviation. The level of significance was set at $P < 0.05$. *Significant difference related to CML; ** significant difference related to LML.

tion (T18 eels), both layers of the tunica muscularis displayed a sharply decreased thickness, with the CML and LML being 56 ± 16 μm ($P < 0.05$) and 32 ± 2 μm ($P < 0.03$), respectively, and the CML/LML ratio equal to 1.6 ($P > 0.2$).

In male eels not treated with hormone, starved for 10 weeks and kept in freshwater, CML and LML thicknesses were 191 ± 6 μm ($P > 0.1$) and 58 ± 12 μm ($P < 0.01$), respectively. The CML/LML ratio was similar to that of control eels (CML/LML 3.4; $P > 0.8$). In contrast, in male eels kept in salty water, CML and LML thicknesses were 51 ± 3 μm ($P < 0.05$) and 35 ± 12 μm ($P > 0.06$), respectively (CML/LML = 1.7; $P < 0.04$) (Fig. 1).

Density of Hu-IR neurons in longitudinal sections and wholemount preparations

Myenteric Hu-IR neurons showed different levels of immunoreactivity, from moderate to very bright, and in wholemount preparations it was possible to observe neurons scattered quite homogeneously (Fig. 2a), although clusters of cells forming large ganglia in the plexus were also visible. In wholemount preparations we measured the density of the total number of small-, medium-, and large-sized Hu-IR neurons and also the density of giant neurons (Fig. 2a-f). As Hu-IR does not label neuronal processes, we could not

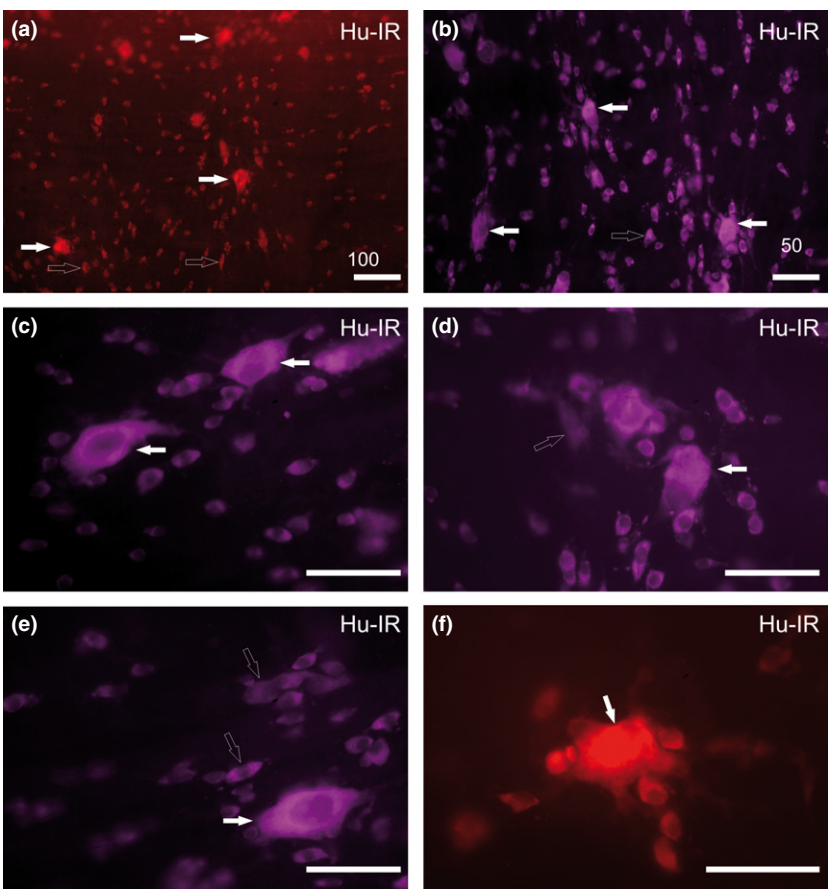

Fig. 2 Wholemount preparations of the myenteric plexus of the cranial portion of freshwater eel intestine showing human neuronal C/D (Hu) immunoreactivity. *White arrows* (a-f) indicate large neurons (giant neurons) with irregular outline of neuronal and cell body (c-e); Hu-IR was also observed at the beginning of the neuronal processes of some giant neurons, showing up to five emerging processes (f). Giant neurons were often encircled by small neurons (f). *Empty arrows* indicate medium-sized neurons, whereas most MP neurons were small. Scale bar: 100 μm (a); 50 μm (b-f).

establish the Dogiel type morphology of the immunostained neurons.

Longitudinal cryosections

To avoid double counting or overestimation of Hu-IR neurons, neuronal cell bodies were counted in serial sections at least 140 μm apart (10 slices). MP neurons were organized in a single-cell layer interposed between the CML and LML. SMP neurons were very rarely observed in the control eels and, where present, these cells occupied different levels of the submucosa. The following paragraphs deal with an accurate quantitative analysis of Hu-IR neurons in the MP and SMP of the five experimental groups examined in this work. The results were crucial to establish the neuroplastic changes that represent the core of the present research.

Control eels. In female freshwater eels the density of Hu-IR MP and SMP neurons was 140 ± 25 and 17 ± 8, respectively; this was similar to the density measured in the MP (137 ± 20; $P > 0.95$) and SMP (27 ± 2; $P > 0.6$) of male subjects (Fig. 3a,c).

T0 eels. In this group of eels, already adapted to brackish water and showing the thickest intestinal musculature, the number of MP and SMP neurons was 183 ± 15 and 18 ± 14, respectively; the number of MP neurons was significantly greater ($P < 0.001$) than that observed in the MP of control eels.

T18 eels. These eels, starved and kept in salty water for a long time, showed the most striking decrease in muscle layers. The number of Hu-IR MP and SMP neurons was markedly increased (225 ± 30 and 61 ± 8, respectively), although only the number of SMP neurons was significantly greater comapred with both control ($P < 0.05$) and T0 eels ($P < 0.05$) (Fig. 3b,d). The higher number of SMP neurons observed in this group (Fig. 3b,d) was linked to a particular organization of ganglia, with a 'chain' of neurons (forming a 'snake-like' column) apparently moving from the MP to the 'submucosa' frequently observed (Fig. 3e,f).

Untreated male eels. In freshwater eels, starved for a shorter time (10 weeks) and showing a moderate decrease in muscle layers, the number of MP and SMP neurons was

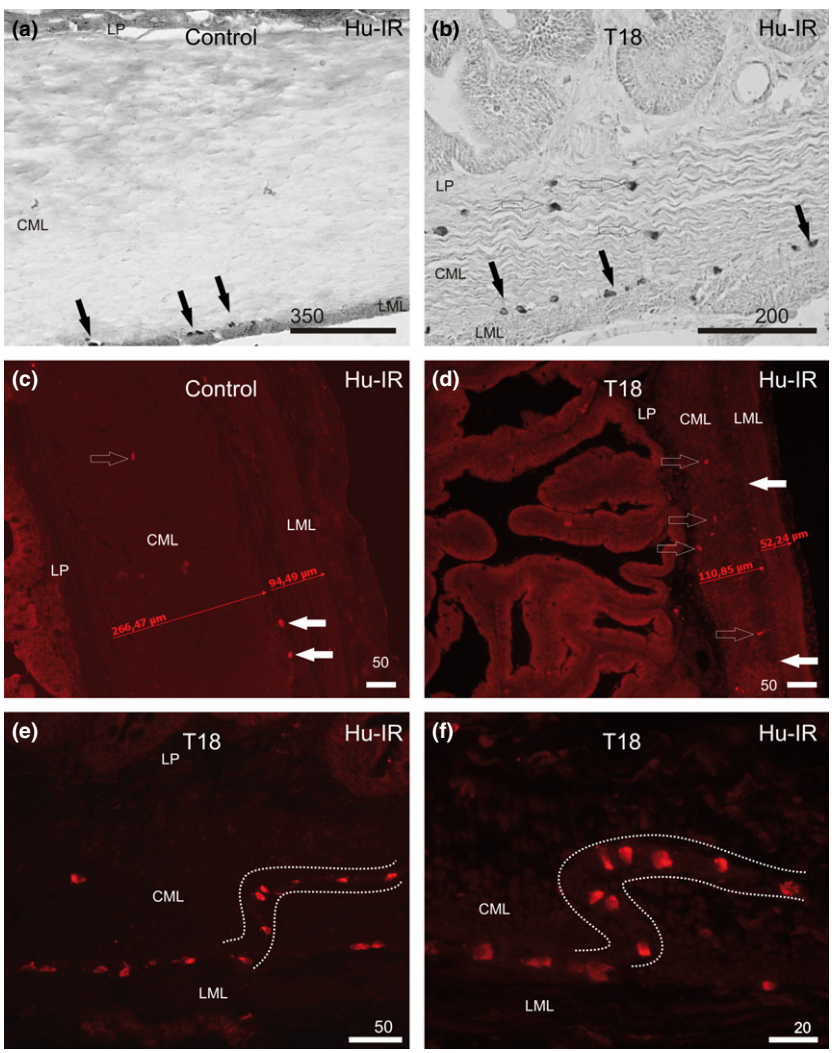

Fig. 3 Cryosections of the cranial portion of the intestine belonging to freshwater (control) and saltwater-adapted (T18) eels (a,d). In control eels the thickness of the circular muscle layer (CML) was greater than in T18 eels, as demonstrated more accurately in (c,d). Black arrows indicate myenteric plexus neurons and empty arrows indicate submucosal neurons. It is evident that seawater-adapted subjects had more SMP neurons. (e–f) Apparent migration of neurons from the myenteric plexus to the CML/submucosa in seawater-adapted ells (T18). Noteworthy, 'snake-like' columns of neurons moving from MP to SMP were often observed (dotted lines). CML, circular muscle layer; LML, longitudinal muscle layer; LP, lamina propria. Scale bar: 350 μm (a); 200 μm (b), 50 μm (c,e), 20 μm (f).

74 ± 2 and 14 ± 3, respectively. Intriguingly, in seawater eels starved for the same time (10 weeks) and showing reduced muscle layer thickness, the number of MP and SMP neurons was 126 ± 15 and 21 ± 4, respectively; in the MP ($P < 0.007$) and SMP ($P < 0.05$) the number of neurons was statistically higher than those observed in male untreated freshwater eels. All data are illustrated in Fig. 4(a,c).

Myenteric plexus wholemount preparations

Control eels. In female eels, there were 162 ± 12 Hu-IR neurons, almost as many as those found in male subjects (168 ± 25); the value for both sexes was 140 ± 23. The average number of neurons in each field of female and male eels was 16 ± 1 and 17 ± 3, respectively, and the value for both sexes was 14 ± 2. Few giant neurons were observed (3 ± 1 and 2 ± 1, respectively), and the value for both sexes was 2 ± 0.6.

T0 eels. There were significantly more Hu-IR neurons (296 ± 41; $P < 0.01$) in T0 eels than in control subjects. The average number of neurons in each field was 27 ± 2 and the number of giant cells 4 ± 1.

T18 eels. The number of neurons (439 ± 38 neurons) was significantly higher ($P < 0.05$) in T18 eels than control eels, but not T0 eels ($P < 0.03$). There were 44 ± 4 neurons in each field and 6 ± 0.7 giant cells.

Starved untreated male freshwater eels. The number of Hu-IR neurons was 284 ± 67, larger ($P < 0.05$) than the number of neurons counted in male control eels (168 ± 25). The average number of neurons in each field was 29 ± 7 and the number of giant cells 8 ± 3.

Seawater starved untreated male eels. In salty water subjects, there were significantly more Hu-IR neurons (350 ± 11) ($P < 0.05$) than observed in freshwater animals. The average number of neurons in each field was 35 ± 1 and the number of giant cells 5 ± 2.

In general, all of the data obtained in wholemount preparations – with the exception of those obtained in starved untreated male freshwater eels – confirmed the results obtained in cryosections. All data are illustrated in Fig. 4(b,d).

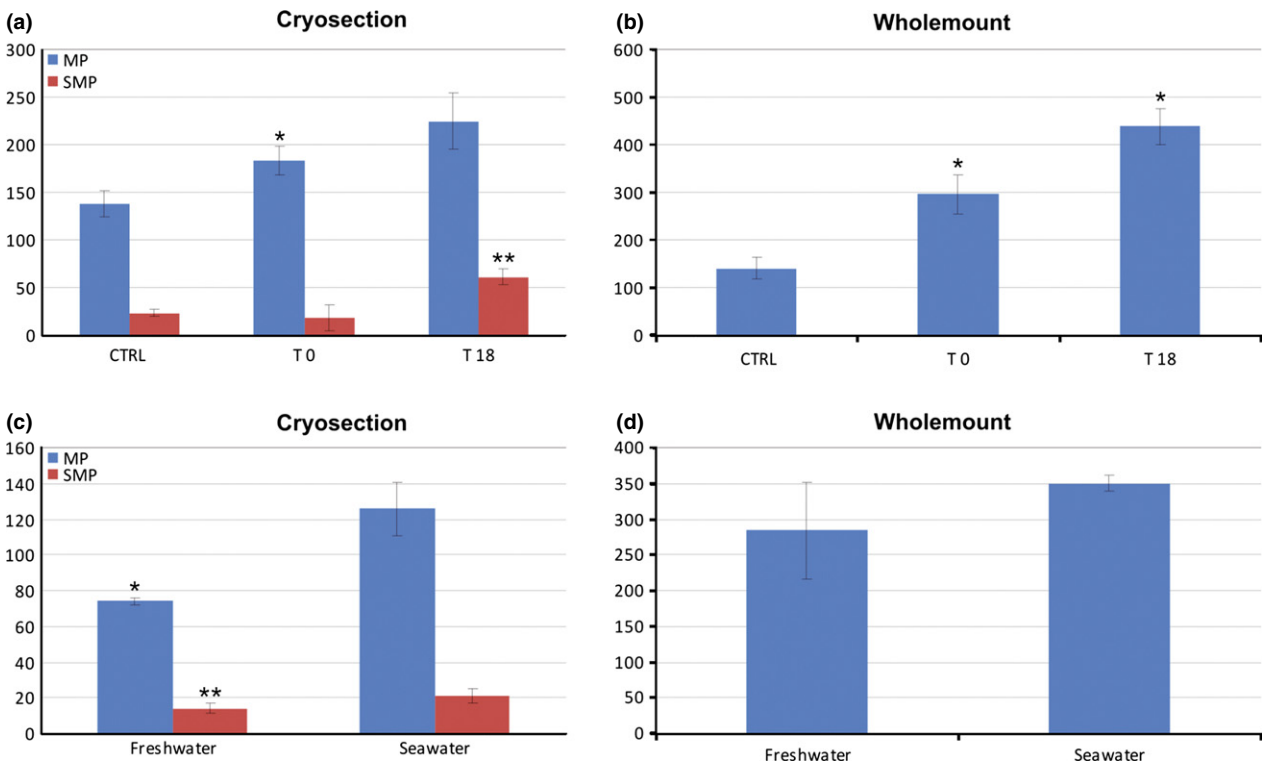

Fig. 4 (a,b). Neuronal density in the cranial portion of the intestine of control (CTRL), T0 and T18 eels. Control subjects were male and female freshwater eels, T0 subjects were female eels captured in salty water lagoons in northern Italy before leaving the coasts for reproductive migration, whereas T18 subjects were female eels captured as T0 subjects and adapted in captivity to seawater salinity for 18 weeks. (a) Histogram showing the number of Hu-immunoreactive myenteric (MP) and submucosal plexus (SMP) neurons counted in 1-cm-long longitudinal cryosections. (b) Histogram showing the number of Hu-immunoreactive myenteric plexus (MP) neurons counted in 10 fields (40×) of wholemount preparations. (c,d) Neuronal density in the cranial portion of the intestine of male eels measured in longitudinal cryosections (c) and wholemount preparations (d). Male eels were subdivided into two hormone-untreated groups and maintained in starvation for 10 weeks in freshwater and seawater. Data are expressed as mean ± standard error of the mean. The level of significance was set at $P < 0.05$. *Significant difference related to the MP; **significant difference related to the SMP.

Enteric glial cells

In control freshwater eels, S100β-IR glial cells, which showed faint to moderate labelling intensity, were more concentrated around MP and SMP neurons, whereas few glial processes were seen between LML and CML outside the myenteric ganglia. Glial cells and processes were present also in the submucosa and lamina propria (Fig. 5a-c).

In T0 and, in particular, in T18 eels, large bundles of strongly labelled S100β-IR glial processes and cells were observed around MP neurons and between the LML and CLM; bright S100β-IR enteric glial cells were seen close to SMP neurons (Fig. 5d,e). Some 'snake-like' columns of Hu-IR neurons were seen from MP to the submucosa, accompanied by S100β-IR glial cells and processes (Fig. 5h-j). Furthermore, glial cells and processes were more evident in the lamina propria of the mucosal folders than in control subjects. Finally, in starved untreated male eels, S100β-immunolabelling showed less density and brightness compared with control eels. However, no differences were seen between eels kept in fresh or salty water.

Myenteric neuron size

Freshwater eels. In control eels, the average cross-sectional area of small, medium and giant neurons was 84 ± 25, 191 ± 53, and 487 ± 127 μm^2, respectively.

T0 eels. Compared with controls, eels ready for spawning migration showed not only a thicker musculature but also larger neurons in all three categories of neurons considered. The average cross-sectional area of small, medium, and giant neurons was 103 ± 38 μm^2 ($P > 0.06$), 201 ± 51 μm^2 ($P > 0.08$), and 629 ± 249 μm^2 ($P > 0.09$), respectively.

T18 eels. After long-time starvation, the cross-sectional area of small- (70 ± 29 μm^2, ($P > 0.06$) and medium-sized neurons (173 ± 91 μm^2, ($P > 0.08$) decreased compared

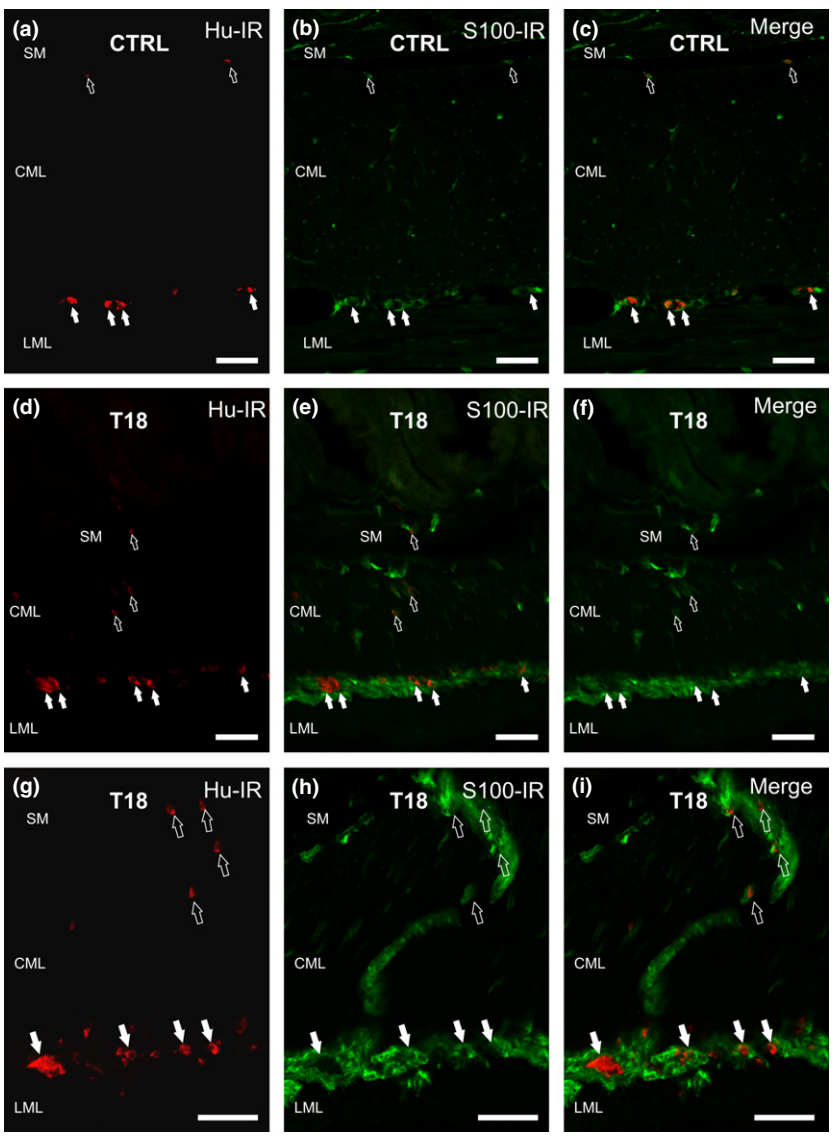

Fig. 5 (a-j) Photomicrographs showing the distribution of enteric neurons immunoreactive for the pan-neuronal marker Hu (Hu-IR) and enteric glial cells immunoreactive for the glial marker S100 (S100-IR) in cryosections of control (CTRL) and T18 eels; control eels were freshwater eels, T18 eels were female eels adapted in captivity to seawater salinity for 18 weeks. (a-c) White arrows indicate myenteric plexus (MP) Hu-IR neurons of CTRL eels encircled by thin processes of enteric S100-IR glial cells, and empty arrows indicate two smaller submucosal Hu-IR neurons. In CTRL eels, glial cells and processes were mainly confined to around MP and SMP neurons and also in the submucosa (SM) and lamina propria. (d-f) In T18 eels, bright S100-IR glial cells and processes were densely distributed around MP and SMP neurons and were also abundantly observed along the space between the longitudinal (LML) and circular (CML) muscle layers. (h-j) In T18 eels, large bundles of enteric S100-IR glial cells and processes were also observed along the 'snake-like' trajectory of migrating neurons moving from the MP to the SMP. Scale bar: 50 μm.

with controls, whereas giant neurons were slightly larger (516 ± 240 μm^2; $P > 0.09$).

Starved untreated male freshwater eels. Compared with controls, these eels, which had starved for a shorter time than T18 ones, presented a smaller area of all three categories of neurons considered. The average cross-sectional area of small, medium, and giant neurons was 72 ± 26 μm^2 ($P > 0.08$), 167 ± 50 μm^2 ($P > 0.09$), and 377 ± 160 μm^2 ($P > 0.06$), respectively.

Starved untreated male salty water eels. In this case as well, all three categories of neurons were smaller than in controls. The average cross-sectional area of small, medium, and giant neurons was 71 ± 17 μm^2 ($P > 0.08$), 184 ± 52 μm^2 ($P > 0.1$), and 383 ± 105 μm^2 ($P > 0.09$), respectively.

Data are illustrated in Fig. 6.

Discussion

The most important finding of the present research is the neuronal plasticity observed in eel ENS of both sexes after 10–22 weeks of modified body homeostasis. The results of the sequence of experiments performed in the present study indicate that salty water adaptation is crucial for the proliferation and/or migration/differentiation of enteric neurons. Notably, male eels that do not receive hormone treatment and are kept in salty water for a few weeks undergo marked changes, characterized by enteric neuroplasticity. In line with this, the main results are the increased density of neurons in the MP and the 'neuronal migration' that leads to a larger number of neurons in the SMP.

Salinity might be a prominent factor evoking enteric neuronal plasticity in eels. This is supported by evidence that, compared with controls, T0 eels (taken from lagoons with mixed fresh and salty water) exhibited increased neuronal density.

Furthermore, wholemount preparations from hormone-untreated starved male eels (salt water > freshwater) showed significantly increased neuronal density compared with controls of the same sex. Likely, starvation in freshwater may also contribute to neuronal remodelling by increasing neuronal density. Nonetheless, the basic mechanisms by which salty water and starvation elicit neuroplasticity, i.e. by affecting the enteric neuronal density, remain unexplained and are, in any case, outside the purpose of this study.

The results of our study open important perspectives in enteric neuroplasticity and the related functional aspects of the ENS, two topics that will be briefly discussed below.

Measurement of the muscular layers thickness

Eels are known to survive for a long time in starving conditions (from 512 days to 4 years; Honma & Matsui, 1972; Boëtius & Boëtius, 1967). Our study showed that long-term starvation elicits a progressive reduction of the muscle layers in seawater-adapted eels (Fig. 3c,d), as previously demonstrated (Honma & Matsui, 1972; Clarke & Witcomb, 1980; Pankhurst & Sorensen,1983; Gisbert et al. 2011). In eels ready for migration (T0 group), CML thickness was greater than that of freshwater eels; the biological meaning of these changes is still unclear, although one might speculate that silver eels eat abundantly before their long migration to the Sargasso Sea (De Leo & Gatto, 1996).

The results regarding muscle thickness changes deserve discussion. Indeed, after 10 weeks of starvation, the musculature of male hormone-untreated eels showed a marked reduction in CML thickness, which was particularly true in eels kept in salty water and comparable to that observed in T18 eels. Likely, salinity could affect muscle layer thickness, although the mechanisms underlying these changes remain obscure and are certainly beyond the purpose of this study. The most striking reduction of muscle layer thickness was obtained after 18 weeks of starvation (T18 female eels) –likely resulting from a combination of factors that include starvation, hormone treatment and salinity of the water.

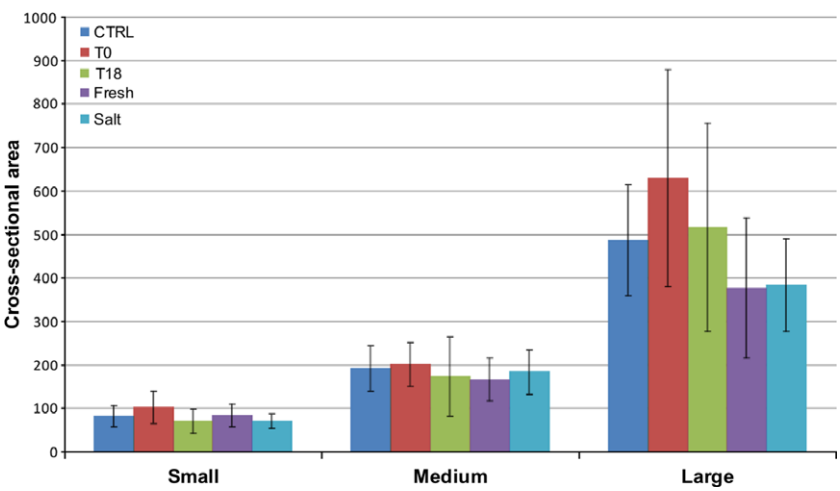

Fig. 6 Graphical representation of the cross-sectional area of small, medium and large neurons measured in wholemount preparations of all the groups of eels considered. CTRL (control), female freshwater eels; T0, female eels captured in lagoons in northern Italy before leaving the coasts for reproductive migration; T18, female eels captured as T0 subjects and adapted in captivity to seawater salinity for 18 weeks; fresh and salt eels, hormone-untreated male eels maintained in starvation for 10 weeks in freshwater and seawater, respectively.

Enteric neuroplasticity/neurogenesis

Studies on fish (Olsson et al. 2008) suggest that only vagal neural crest cells contribute to ENS formation. In the present research we observed an increased number of Hu-IR neurons in both enteric plexuses of eels kept in salty water. Apparently, new SMP neurons originate and/or migrate from the MP, as it was quite common to observe neurons and associated glial cells arising from the MP and directed (in a 'chain-like' pattern) to the submucosa. These neuroplastic changes led us to speculate on the possible role of stem cells resident in the ENS. Neuronal elements able 'to react' to mechanical and chemical stimuli have long been demonstrated to exist in the ENS (Filogamo & Cracco, 1995; Hanani et al. 2003; Vasina et al. 2006). However, only recent data have provided evidence of enteric stem cells committed to the neural line and able to differentiate into neurons or glial cells (Kruger et al. 2002; Laranjeira et al. 2011). Furthermore, it has been shown that the adult ENS may have stem cells that are the origin of enteric neurons *in vitro* (Bixby et al. 2002; Bondurand et al. 2003) and *in vivo* (Liu et al. 2009). Although there is still debate about the exact location of enteric undifferentiated neuronal precursors, there is the possibility that progenitors of such newly generated neurons may reside in germinal niches close to myenteric plexuses or along the mesentery. Recently, it has been demonstrated that enteric neuronal progenitors of adult mice express glial markers and that these cells retain a neurogenic potential, being capable of generating enteric neurons in culture (Laranjeira et al. 2011). These data support the possibility that resident neural stem cells can differentiate in enteric neurons and glial cells in response to water salinity. Whether these neurons exist and differentiate in our model and what their functional role is in eel gastrointestinal physiology are all aspects that need to be elucidated.

We observed an interesting, and apparently contradictory, result in starved and hormone-untreated male eels kept in fresh and salty water, where, against a background of increased neuronal density, we failed to observe a consequent S100β-IR enhancement. As serum levels of S100 protein decreased in chronic starvation and normalized with weight gain (Holtkamp et al. 2008), it is plausible that in starved male (hormonally untreated) eels, a reduction of S100 protein occurs at the tissue level in the ENS.

Effects of enteric neurons on mucosal functions

In teleost fishes, SMP neurons may be absent (Harder, 1975) or poorly represented (Karila & Holmgren, 1995; Wallace et al. 2005; Olsson, 2009). The paucity of SMP neurons might be correlated to the absence of the muscularis mucosae; in fact, in the *Salmo gairdneri* trout, SMP neurons are present only in the caudal portion of the intestine, the only gut portion where the muscularis mucosae is well developed (Ezeasor, 1979). Due to the paucity of neurons in the SMP of freshwater eels, MP neurons are very likely to be involved in the regulation of mucosal function. In this respect, in the eel, the oesophagus may play a major role in osmoregulation (Ando et al. 2003; Grosell, 2006), along with the middle portion of the intestine, followed by the posterior and anterior parts. There are contradictory data concerning the role of the rectum (Ando & Kobayashi, 1978; Ando, 1980; Kim et al. 2008). The first structural modifications of the eel intestine start 3 days after adaptation to seawater (MacKay & Janicki, 1978; Yamamoto & Hirano, 1978), whereas intestinal Na^+/K^+-ATPase activity increases after 3 weeks. In fact, water absorption across the intestine requires increased activity, expression levels and possibly changes in subunit isoforms of Na^+/K^+ ATPase pumps (Laverty & Skadhauge, 2012). Intestinal water absorption is clearly driven by active transepithelial NaCl transport; the major players are the basolateral Na^+/K^+-ATPase and a number of apical transporters, including Na^+-Cl^- and Na^+-K^+-$2Cl^-$ co-transporters (Grosell, 2006). Based on this study, it is conceivable that enhanced gut physiological functions following adaptation to salty water require the recruitment of ENS precursors in the eel gastrointestinal tract (GIT) (Cooke, 1989).

Effects of starvation on enteric neuronal plasticity

Contradicting data have been reported concerning the effect of starvation on the density (and neurochemistry) of enteric neurons in mammals (Shochina et al. 1997; Toole et al. 1999). In general, malnutrition or protein deprivation studies demonstrated that the number of enteric neurons did not decrease in the rat small intestine (Natali et al. 2003; Moreira et al. 2008), and caloric restriction appears to have a protective effect on myenteric neurons during aging (da Silva et al. 2012). Our data suggest that starvation might enhance MP neuronal density (starved untreated freshwater eels vs. control eels).

Effects of hormonal treatment on neuronal plasticity

A growing body of literature describes the effects of hormonal/pheromonal treatment on neuronal plasticity of defined portions of the mammalian and fish CNS (Chung-Davidson et al. 2008; Fowler et al. 2008; Lieberwirth & Wang, 2012). The carp pituitary extract contains a mix of hormones, including gonatrotopins, adrenocorticotropic hormone (ACTH), thyroid-stimulating hormone, growth hormone (GH), and others, which might play a role on the presumed neuronal stem cells differentiation implied above (Li et al. 2011; Waters & Blackmore, 2011). As an example of the effects mediated by hormones on neurogenesis, it has been shown that the 17-β estradiol is a potent modulator of physiological neurogenesis, especially in the hippocampus of mammals (Li et al. 2011). Thus, due

to gonadotrophic treatment in this study, it is possible that enteric neurogenesis in female eels was affected by the 17-β estradiol released as a result of induced steroidogenesis of the oocyte follicle layers. Indeed, in the *Anguilla japonica*, ACTH and glycocorticoid have been shown to enhance the intestinal uptake of Na^+, Cl^- and water through the activity of Na^+-K^+ ATPase (Hirano & Utida, 1968, 1971; Yamamoto & Hirano, 1978). How these hormones act on ATPase activity or on the enteric neurons controlling these functions is still unknown. Cortisol and, consequently, stress seem to reduce cell proliferation in the telencephalon of fish (rainbow trout) (Sørensen et al. 2011, 2012) but at present there are no data available regarding the ENS.

The dimension of the myenteric neurons

There are few investigations related to the morphometry of fish (and mammals) enteric neurons available. Our data may be compared with those obtained by Olsson on shorthorn sculpin (*Myoxocephalus scorpius*; Olsson, 2011a) and zebrafish (*Danio rerio*; Olsson, 2011b). In the shorthorn sculpin it has been shown that MP neurons of the cranial intestine have a mean cross-sectional soma area of 240 ± 177 μm^2 (data expressed as average ± standard deviation), whereas in the zebrafish the mean cross-sectional area was 93 ± 3 μm^2. We should take into consideration that the two species are very different in size and that neurons represent an exception to 'Driesch's law'.

No significant differences were observed in the size of the subclasses of neurons measured in the five groups of eels. Nevertheless, T0 eels, i.e. eels prepared for long migration and which showed the thickest muscular layers, had also the largest neuronal dimension, especially in giant cells, neurons immunoreactive for nNOS-IR (data not shown), which are probably involved in muscle control. Giant neurons are smaller after 10 weeks of starvation in untreated male eels but, unexpectedly, they are preserved in female eels after 18 weeks of starvation.

Critical consideration of Hu-immunostaining and other possible biases of this study

The enteric neuronal cell bodies were identified with the anti-Hu antiserum that recognizes RNA-binding proteins of the embryonic lethal abnormal vision (Elav) family, which are conserved across vertebrate species (Okano & Darnell, 1997; Inman et al. 1998). The Hu proteins (especially HuC/D) are recognized as one of the earliest markers of the neuronal phenotype (Perrone-Bizzozero & Bolognani, 2002; Deschênes-Furry et al. 2006), as they suppress neuroblast proliferation and promote neuronal differentiation (Marusich et al. 1994; Okano & Darnell, 1997; Akamatsu et al. 1999). Antibodies to HuC/D have been extensively used to label all enteric neurons in mammals (Fairman et al. 1995; Chiocchetti et al. 2004; Hoff et al. 2008), birds (Marusich et al. 1994; Barami et al. 1995) and fishes (Bernardos & Raymond, 2006; Olsson, 2011a,b). Studies using HuD knockout mice showed an increased ability of progenitor cells to renew, along with a decreased capacity to differentiate into neurons (Akamatsu et al. 2005). Thus, it is likely that we missed possible progenitors by using HuC/D labelling to identify and quantify enteric neurons in the eel gastrointestinal tract.

Counting the density of neurons in the ENS is always a difficult challenge. Wholemount preparations might be criticized, as the intestinal tract of starved animals presents a minor extension and consequently an apparently increased neuronal density. To obviate this problem, the neuronal counting was performed in wholemount preparations along with longitudinal cryosections, which allowed us to evaluate the density of neurons in the 'submucosa'. The data of neuronal counting on MP wholemount preparation (taken alone) might be questionable; however,the combination of neuronal density data obtained from MP and longitudinal sections for SMP neuronal density, showing groups (or 'chain') of neurons possibly derived from the MP, provides evidence of an increased number of neurons. This reflects possible enteric neuroplastic mechanisms occurring in the eel gut in response to salty water adaptation.

In conclusion, our study indicates major neuronal and muscular remodelling as a result of adaptive plasticity in the eel gastrointestinal tract. Likely, salty water and other environmental factors may contribute to these changes. The discovery of the molecular mechanisms underlying the neuro-muscular adaptive changes observed in this unique model will pave the way to a better understanding of enteric neuro-muscular plasticity.

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Author contributions

Study concept and design: R.C., P.C., O.M. and A.D.B. The *in vivo* experiments were performed in the facilities and labs of A.P., O.M., A.D.B. and R.S. The immunohistochemical experiments were carried out by C.S., R.C., O.P. and V.M. Acquisition of data: R.C., O.P. and C.S. Drafting of the manuscript: R.C. Critical revision of the manuscript for important intellectual content: R.DeG. Statistical analysis: C.S. All authors contributed to the revision of the article for critical intellectual content and approved the final version. Funding sources: R.DeG., P.C. and O.M. Study supervision: R.C. and R.DeG.

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